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A *Plasmodium falciparum* Transcriptional Cyclin-Dependent Kinase-Related Kinase with a Crucial Role in Parasite Proliferation Associates with Histone Deacetylase Activity^{∇†‡}

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Cyclin-dependent protein kinases (CDKs) are key regulators of the eukaryotic cell cycle and of the eukaryotic transcription machinery. Here we report the characterization of Pfcrk-3 (*Plasmodium falciparum* CDK-related kinase 3; PlasmoDB identifier PFD0740w), an unusually large CDK-related protein whose kinase domain displays maximal homology to those CDKs which, in other eukaryotes, are involved in the control of transcription. The closest enzyme in *Saccharomyces cerevisiae* is BUR1 (bypass upstream activating sequence requirement 1), known to control gene expression through interaction with chromatin modification enzymes. Consistent with this, immunofluorescence data show that Pfcrk-3 colocalizes with histones. We show that recombinant Pfcrk-3 associates with histone H1 kinase activity in parasite extracts and that this association is detectable even if the catalytic domain of Pfcrk-3 is rendered inactive by site-directed mutagenesis, indicating that Pfcrk-3 is part of a complex that includes other protein kinases. Immunoprecipitates obtained from extracts of transgenic parasites expressing hemagglutinin (HA)-tagged Pfcrk-3 by using an anti-HA antibody displayed both protein kinase and histone deacetylase activities. Reverse genetics data show that the *pfcrk-3* locus can be targeted only if the genetic modification does not cause a loss of function. Taken together, our data strongly suggest that Pfcrk-3 fulfils a crucial role in the intraerythrocytic development of *P. falciparum*, presumably through chromatin modification-dependent regulation of gene expression.

Plasmodium falciparum, the protozoan parasite responsible for the most virulent form of human malaria, causes 1 to 3 millions deaths annually, mostly among children in sub-Saharan Africa. This mortality is expected to rise with the global emergence and spread of drug-resistant parasites, making the discovery of alternative control agents an urgent task (43). The identification of potential targets is now greatly facilitated by the availability of genomic databases for several species of the *Plasmodium* genus (www.plasmoDB.org) (49). *Plasmodium* cell cycle regulators represent attractive candidate targets for intervention, because (i) their activities are most probably essential to parasite survival, and (ii) the overall organization of the cell cycle in malaria parasites differs considerably from that

in mammalian cells; this is reflected by atypical properties of the enzymatic machinery controlling cell cycle progression, suggesting that specific inhibition is achievable (12, 15).

The progression of the eukaryotic cell cycle is tightly controlled by a family of protein kinases, the cyclin-dependent kinases (CDKs), whose active forms are composed of a catalytic subunit (CDK) and a regulatory subunit (cyclin) (39). While several mammalian CDKs (CDK1, -2, -3, -4, -6, and -7) function in cell cycle control, others (CDK8, -9, -10, and -11) are part of the transcription machinery. CDK7 is a regulator both of cell cycle progression (through its activity as a CDK-activating kinase [CAK]) and of transcription (through its activity as a component of the general transcription factor TFIIF) (26). CDK8 and -9 regulate transcription by phosphorylating the C-terminal domain of the large subunit of RNA polymerase II (2, 18); BUR1, the *Saccharomyces cerevisiae* CDK9 homologue previously known as SGV1, has been shown to regulate transcription through selective control of histone modifications (7, 17, 30). CDK10 regulates transcription and cell cycle progression by modulating the activity of the Ets2 transcription factor, a regulator of CDK1 expression (27). CDK11 interacts with the general precursor mRNA splicing factors and with RNA polymerase II, thereby playing a role in transcript production and the regulation of RNA processing (37). Finally, CDK5 has neuron-specific functions (11).

Among the 85 (or 99, depending on the criteria used for

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inclusion) eukaryotic protein kinase (ePK) sequences that were identified in the *P. falciparum* kinome (3, 52), 18 clustered within the CMGC group (CDKs, MAPKs [mitogen-activated protein kinases], GSK3 [glycogen synthase kinase 3], and CDK-like), with 6 sequences more closely related to CDKs than to other CMGC subfamilies. By analogy with their functions in other eukaryotes, and despite the unique characteristics of the *Plasmodium* cell cycle (13, 31, 41) and transcription machineries (1, 6, 8–10), it is likely that the *Plasmodium* CDK-related kinases play key roles in cell cycle progression and transcription in the parasite. Among those gene products, PfPK5 (22, 32, 47), Pfcrk-1 (14), Pfmrk (34, 35, 53), and PfPK6 (5) have been the subjects of biochemical or structural investigations. However, the only reverse genetics-based information published so far regarding the function of CDKs in the parasite life cycle is that for Pfcrk-1, the orthologue of Pfcrk-1 in *Plasmodium berghei*, which is essential for erythrocytic schizogony (46).

Here we report the functional characterization of *pfcrk-3* (PlasmoDB identifier PFD0740w), a gene encoding an unusually large CDK-related protein (1,339 amino acids) whose kinase domain displays maximal homology to those CDKs which, in other eukaryotes, are involved in the control of transcription. The enzyme associates with a kinase activity present in parasite extracts, and this association is detectable even if the catalytic domain of Pfcrk-3 is rendered inactive by site-directed mutagenesis, suggesting that Pfcrk-3 is part of a complex containing other protein kinases. We demonstrate that Pfcrk-3 interacts with a histone deacetylase (HDAC) in parasite extracts, and we provide reverse genetics evidence strongly suggesting that the *pfcrk-3* gene plays a crucial role in parasite proliferation during the asexual erythrocytic cycle.

MATERIALS AND METHODS

GST-Pfcrk-3 expression plasmid and site-directed mutagenesis. The Pfcrk-3 catalytic domain was amplified from the 3D7 cDNA clone by using oligonucleotides carrying a BamHI (forward primer, CGGGGATCCGATAAAAGAATGTAAGTTACACA) or a SalI (reverse primer, GGGGTCTGACTTATCTTTTGATTACTCTGT) site (underlined). The PCR product was inserted into the pGEX4T3 plasmid (Amersham Biosciences) at the BamHI and SalI sites. GST-Pfcrk-3-K445M, a plasmid encoding a mutant glutathione *S*-transferase (GST)-Pfcrk-3 fusion protein with an alteration from lysine to methionine at residue 445, was obtained by site-specific mutagenesis using the overlap extension PCR technique (21). The plasmids were electroporated into *Escherichia coli* strain BL21, and the inserts were verified by DNA sequencing prior to protein expression.

Expression and purification of recombinant proteins. GST, GST-Pfcrk-3, and GST-Pfcrk-3-K445M were induced in *Escherichia coli* (strain BL21 codon+) with 0.5 mM isopropyl- β -thiogalactopyranoside at 30°C for 4 h. Cells were harvested and resuspended in ice-cold sonication buffer (phosphate-buffered saline [PBS] [pH 7.5], 0.1% Triton, 1 mM EDTA, 1 mM dithiothreitol [DTT]) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and Complete mixture inhibitor tablet from Roche) and 100 μ g/ml lysozyme. After 10 min on ice, the suspension was sonicated and clarified by centrifugation at 11,000 \times g for 30 min at 4°C. The resulting supernatant was incubated with glutathione Sepharose resin (Sigma) for 1 h. The resin was washed four times with sonication buffer and once with a buffer containing 50 mM Tris-HCl (pH 8.7)–75 mM NaCl. The protein concentration was determined using the Bio-Rad dye reagent according to the manufacturer's recommendations with bovine serum albumin as a standard. Aliquots of purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining.

Parasite culture and preparation of parasite extracts. The *P. falciparum* clone 3D7 was cultured *in vitro* by standard methods (25). The parasites were grown in human erythrocytes at 5% hematocrit in complete RPMI 1640 medium in 25-cm² ventilated flasks. The flasks were kept in a 37°C incubator under 5% CO₂. To

remove serum and leukocytes, the human blood was washed three times in RPMI 1640 and the buffy coat removed. The medium was changed daily. Parasitemia was measured daily by examining Giemsa-stained blood smears and was kept between 0.5% and 10%.

Fresh or frozen saponin-lysed *P. falciparum* (3D7) pellets were sonicated in radioimmunoprecipitation assay (RIPA) buffer (30 mM Tris [pH 8.0], 150 mM NaCl, 20 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 10 μ M ATP, 0.5% Triton X-100, 1% NP-40, 10 mM β -glycerophosphate, 10 mM NaF, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 mM benzamide, and Complete protease inhibitors). Lysates were cleared by centrifugation at 10,000 \times g for 15 min at 4°C, and the total amount of protein in the supernatant was measured by the Bio-Rad protein assay.

Pulldown experiments. Glutathione-agarose beads coated with GST, GST-Pfcrk-3, or GST-Pfcrk-3-K445M were incubated in parasite extracts or in RIPA buffer alone at 4°C under mild agitation for 1 h (100 μ g of total parasite proteins for 10 μ g of recombinant proteins on beads). The beads were then washed three times in RIPA buffer, once in RIPA buffer with 0.1% SDS, and once in a standard kinase buffer containing 10 mM sodium fluoride, 10 mM β -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, and Complete mixture protease inhibitors. Beads were resuspended in a volume of kinase buffer equal to the volume of beads. A standard kinase assay was then performed in a final volume of 30 μ l, and the samples were analyzed by SDS-PAGE and autoradiography.

Kinase assays. Kinase assays were performed as described previously (32). Briefly, reactions (30 μ l) were performed in a standard kinase buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 2 mM MnCl₂, 10 mM ATP, and 5 μ Ci [γ -³²P]ATP, using 0.5 μ g of recombinant kinase (or immunoprecipitated material [see below]) and 5 μ g of substrate (myelin basic protein [MBP] or histone H1). After 30 min at 30°C, the reaction was stopped by the addition of Laemmli buffer, and the reaction product was loaded onto a 12% SDS-polyacrylamide gel. Following Coomassie blue staining, the gels were dried and exposed for autoradiography.

Antibody production, immunoprecipitation, and immunofluorescence analyses of wild-type Pfcrk-3. Chicken immunoglobulin (IgY) antibodies against peptides H2N-CKNRRTLNEEDMLSVVD-CONH2 (named VVD) and H2N-PNERDIKTLRNLPCNTN-CONH2 (named PNG), derived from protein sequences (residues 539 to 553 and 840 to 855, respectively, encoded by the PFD0740w gene), were synthesized by Auspep, coupled to rabbit albumin carrier, and inoculated into chickens (the PNG peptide was derived from an early version of PlasmoDB, and the sequence was subsequently changed to PNERDIKTLRNLPCWN; the two substitutions appear not to have prevented recognition of Pfcrk-3 by the antibodies [see Results]). The antibodies were isolated and affinity purified on a peptide-affinity matrix as described previously (19). The IgYs were used in immunoprecipitation as described previously (38). Briefly, an anti-Pfcrk-3 antibody bound to protein A Sepharose beads was incubated with an extract from a parasite at a specific stage (late trophozoite or schizont). Because of the low affinity of chicken antibodies for protein A Sepharose beads, the antibody was first incubated with a rabbit anti-chicken antibody before being coupled to protein A Sepharose beads. After incubation, protein A Sepharose bead-bound complexes were washed and assayed for kinase activity.

For immunofluorescence analysis, *P. falciparum*-infected erythrocytes were fixed with 4% paraformaldehyde and 0.0075% glutaraldehyde as described previously (51). The cells were probed with chicken anti-Pfcrk-3 (1:400) and mouse anti-histone (1:500; Chemicon International) and were subsequently incubated with Alexa Fluor 488-conjugated goat anti-chicken IgY (Molecular Probes) and Alexa Fluor 555-conjugated goat anti-mouse IgG (Molecular Probes). Confocal images were acquired using a laser-scanning microscope (LSM 510; Carl Zeiss).

Immunoprecipitation of HA-tagged Pfcrk-3. Parasites expressing hemagglutinin (HA)-tagged Pfcrk-3 or wild-type 3D7 parasites were obtained by saponin lysis and were then solubilized in M-PER protein extraction reagent (Pierce) containing 25 U/500 μ l Benzamide and a protease inhibitor cocktail (Roche) for 20 min at 4°C. The lysates were cleared at 9,000 \times g and 4°C for 5 min prior to immunoprecipitation (IP). For each IP, 500 μ g protein lysate (in a 100- μ l total volume) was incubated with 10 μ l anti-HA agarose slurry (Pierce) overnight at 4°C and was then washed three times, for 5 min each time, with 500 μ l of cold TBS (25 mM Tris, 0.15 M NaCl [pH 7.2]) buffer.

Histone deacetylase assay. To detect HDAC enzyme activity in the Pfcrk-3 complex, duplicate samples were immunoprecipitated as described above, followed by a final wash in HDAC assay buffer (Millipore Corporation). The beads were further incubated for 16 h at 37°C in 60 μ l HDAC assay buffer containing 100 μ M acetylated fluorogenic peptide and 500 μ M NAD cofactor with or without HDAC inhibitors (10 mM nicotinamide and/or 2 mM trichostatin A). To test for the presence of NAD-independent HDAC activities in the Pfcrk-3 immunoprecipitates, duplicate experiments were also performed in the absence

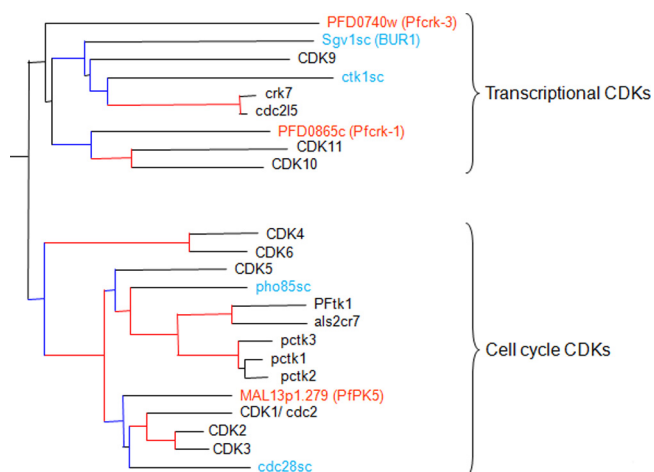


FIG. 1. Three-species phylogenetic tree of CDKs. Sequences in red, blue, and black characters are from *P. falciparum*, *S. cerevisiae*, and *Homo sapiens*, respectively. Branches with bootstrap values of >70 are shown in red, and those with bootstrap values of >40 are shown in blue. (Adapted from reference 52.)

of the cofactor. The beads were pelleted at $9,000 \times g$ for 30 s, and 40 μ l of the supernatant was transferred to each well of a half-volume plate. Twenty microliters of activator solution was added for 15 min, and the resulting fluorescence was recorded using a plate reader (excitation filter, 360/40 nm; emission filter, 460/40 nm).

Transfection plasmids. (i) **pCAM-BSD-*crk-3***. A 1,092-bp DNA fragment (nucleotides 1277 to 2368 of the *pfcrk-3* open reading frame [ORF]) was amplified by PCR from *P. falciparum* genomic DNA, using primers (forward, GGGGGG ATCCGCATATGGAGATGTTTGGATGGC; reverse, GGGGCGGCCCGCTG GTGGTCTATACCATAATGTAATAACTC) containing BamHI or NotI sites (underlined). The amplicon was digested with BamHI and NotI and was inserted at these sites into the pCAM-BSD vector (48).

(ii) **pCAM-BSD-*crk-3*-HA**. The pCAM-BSD-HA vector was generated by introducing a sequence encoding a single HA tag and the 3' untranslated region (3' UTR) of the *P. berghei dhfr-ts* gene into the multiple cloning site of pCAM-BSD (see Fig. 5A). The 3' end of the *Pfcrk-3* coding region (652 bp, omitting the stop codon) was amplified by PCR from genomic DNA, using primers with PstI or BamHI restriction sites, which allowed insertion of the amplified product into the pCAM-BSD-HA vector.

Parasite transfection and genotype characterization. Ring-stage parasites were electroporated with 60 μ g of plasmid DNA (pCAM-BSD-*crk-3* or pCAM-BSD-*crk-3*-HA) as described previously (16). Blasticidin was added to a final concentration of 2.5 μ g/ml 48 h after transfection. Resistant parasites appeared 4 to 5 weeks posttransfection and were cloned by limiting dilution.

For PCR detection of (i) the integration of the pCAM-BSD-*crk-3*-HA plasmid at the 3' flank of the insert, (ii) the integration of the pCAM-BSD-*crk-3* knockout plasmid, and (iii) the episome, the following primers were used to amplify products from total DNA isolated from parasite lines (see below): OL-1 (GGTTCATCAAGTTGGACAAGGAGG), OL-2 (CACAACTCCACATATCAACCGATGC), OL-3 (TATTCCTAATCATGTAAATCTTAAA), OL-4 (CAATTAACCTCACTAAAG), and OL-5 (GAAAGGCTTATCTTCGAAGTA). Primers OL-1, OL-2, and OL-5 correspond to *pfcrk-3* sequences, while primers OL-3 and OL-4 correspond to pCAM-BSD vector sequences flanking the insertion site.

For Southern blot analysis, total DNA was obtained as follows. Parasite pellets obtained by saponin lysis were resuspended in PBS and were treated with 150 μ g/ml proteinase K and 2% SDS at 55°C for 2 h. The DNA was precipitated with ethanol and 0.3 M sodium acetate after phenol-chloroform-isoamyl alcohol (25:24:1) extraction. The DNA was digested with PstI and SmaI, transferred to a Hybond N membrane, and hybridized to the pCAM-BSD or *pfcrk-3* probe.

RESULTS

Bioinformatic analysis of *Pfcrk-3*. Phylogenetic analysis of the *P. falciparum* kinome (3, 52) identified 18 protein kinases

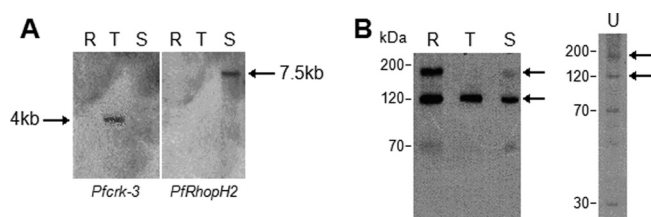


FIG. 2. Expression of *Pfcrk-3* in asexual blood stages. (A) Northern blot analysis. Total RNA was extracted from synchronized 3D7 parasites (R, rings; T, trophozoites; S, schizonts) and subjected to Northern blot analysis. (Left) *pfcrk-3* probe (catalytic domain); (right) *pfirhop2* probe. (B) Western blot analysis. (Left) The immunopurified IgY antibody against VVD (a peptide derived from the *Pfcrk-3* catalytic domain) was used to probe extracts from rings, trophozoites, and schizonts. (Right) An extract from unsynchronized asexual parasites (U) was probed with immunopurified IgY directed against PNG, the large insertion within the *Pfcrk-3* catalytic domain.

belonging to the CMGC group, including *Pfcrk-3*. Among the different families that constitute the CMGC group, *Pfcrk-3* clearly clusters within the CDK family, and more precisely with the CDK8 to -11 group, which comprises CDKs involved in transcriptional control (Fig. 1). BLASTP analysis confirmed the relatedness of *Pfcrk-3* to transcriptional CDKs, with the BUR1 enzyme from yeast giving the highest score (see Discussion). The 11 “invariant” residues that are conserved in most protein kinases (20, 29) are present in *Pfcrk-3*, as are the two signatures that define membership in the protein kinase family (4) (see Fig. S1 in the supplemental material). The polypeptide conforms with a high score to the Pfam protein kinase domain (Pfam entry PF00069; E value, $3.6e-77$).

The predicted *Pfcrk-3* polypeptide (1,339 amino acids) is unusually large for a CDK, because the putative catalytic domain contains two insertions (of 198 and 20 amino acids), and there are large N-terminal (378 residues) and C-terminal (335 residues) extensions (see Fig. S1 in the supplemental material). The extensions and insertions are rich in low-complexity regions and do not display homology to any characterized motif. The cyclin-binding motif (PSTAIR in CDK1, PITALRE in CDK9, and PITQLRE in yeast BUR1) is replaced by AKTYIRE in *Pfcrk-3*. The Thr14 and Tyr15 residues (human CDK2 numbering) are the targets of negative regulation by phosphorylation in several mammalian CDKs; only the Tyr15 residue is present in *Pfcrk-3*, since Thr14 is replaced by an Ala residue. Thr160, which is the target of activating phosphorylation by CDK-activating kinases (CAKs), is conserved in *Pfcrk-3*.

Expression of *pfcrk-3* mRNA and protein in blood stages. Reverse transcription-PCR (RT-PCR) using primers flanking the catalytic domain showed that (i) *Pfcrk-3* mRNA is present in asexual and sexual blood stages, (ii) the gene structure (2 exons separated by a 100-bp intron) proposed in PlasmoDB is correct, and (iii) transcribed mRNA includes the predicted N- and C-terminal extensions (see Fig. S2 in the supplemental material).

Microarray data available on PlasmoDB (33) indicate that *pfcrk-3* mRNA is detectable throughout the asexual cycle (as well as in sporozoites and gametocytes). Northern blot analysis allowed the detection of a single 4-kb *pfcrk-3* mRNA species in trophozoites, although the signal was very weak (Fig. 2A). Probing of the same membrane with *pfirhop2* (a gene ex-

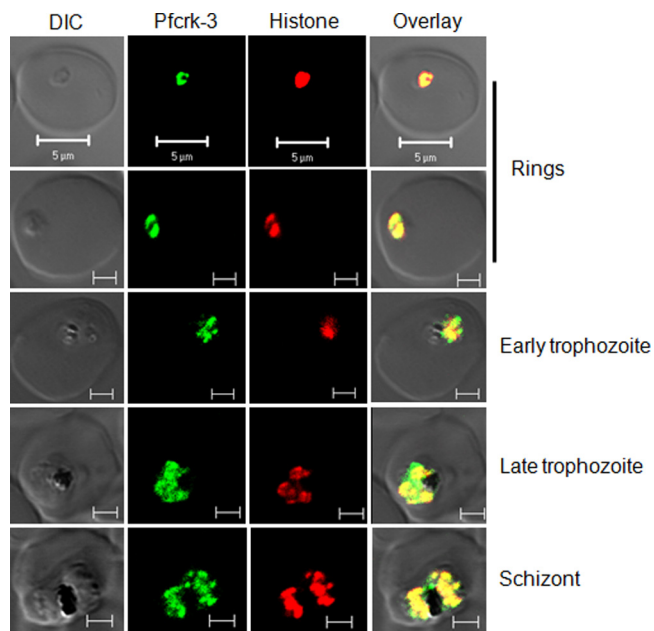


FIG. 3. Pfcrk-3 colocalizes with histones. Images captured by laser scanning confocal microscopy show substantial colocalization of Pfcrk-3 (green) with nucleolar localized histones (red). *P. falciparum* 3D7 parasites were fixed with 4% paraformaldehyde–0.0075% glutaraldehyde and were probed with chicken anti-Pfcrk-3 IgY and mouse anti-histone IgG antibodies. The data in each row represent the fluorescence profile in ring-stage parasites, early trophozoites, late trophozoites, or schizonts, as indicated. DIC, differential interference contrast. Bars, 5 μ m in the top row and 2 μ m in all other rows.

pressed in schizonts) yielded a 7.5-kb mRNA species only in the schizont stage, as expected (36); equal loading was ascertained by ethidium bromide staining, which yielded rRNA bands of similar intensities in all lanes (data not shown).

IgY antibodies against two peptides derived from Pfcrk-3 (one in the large insertion and the other in the catalytic domain [see Fig. S1 in the supplemental material]) recognized recombinant Pfcrk-3 in Western blots (data not shown) (see below for a description of the recombinant protein). Western blotting performed with extracts from synchronous parasites using the anti-VVD antibody (directed against the catalytic domain) showed that although Pfcrk-3 mRNA was detectable predominantly during early stages, the protein was detectable throughout the asexual cycle (Fig. 2B). Pfcrk-3 appears to be proteolytically processed from a large precursor in rings, whose size approximates the expected molecular size of the full-length protein (160 kDa), to a protein of around 120 kDa at later stages, with lower-intensity bands (including one at 70 kDa) also detectable (Fig. 2B, left). Determination of the exact processing events would require additional studies using antibodies directed against the various parts of the protein. The antibody directed against the largest insertion in the catalytic domain (anti-PNG antibody) (Fig. 2B, right) yielded a similar profile, suggesting that processing consists of removal of the extensions.

In view of the clean Western blot pattern obtained with the anti-VVD antibody, we next performed immunofluorescence analysis. Consistent with the Western blot data, the Pfcrk-3

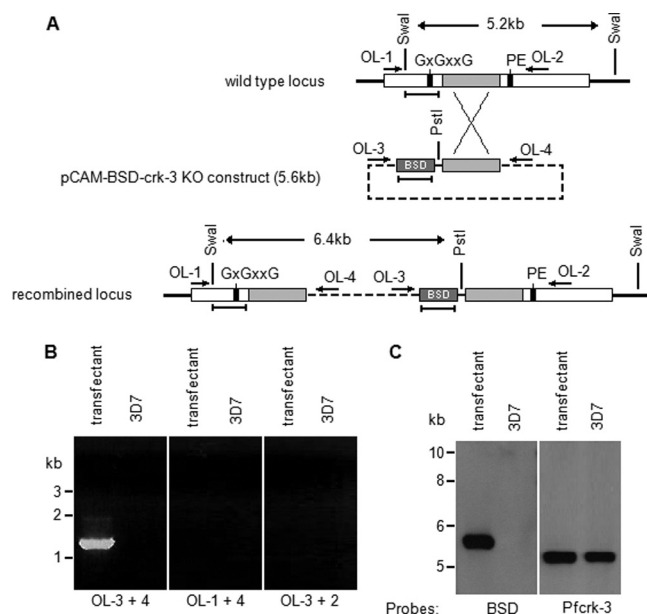


FIG. 4. Attempt at disrupting the *pfcrk-3* gene. (A) Strategy for disruption of the *pfcrk-3* gene. The transfection plasmid contains a PCR fragment spanning positions 1277 to 2368 of the 4.2-kb *pfcrk-3* coding sequence. This fragment excludes two kinase subdomains essential for activity, labeled GxGxxG (a glycine-rich region required for correct orientation of ATP) and PE (a proline-glutamate motif in which the latter residue is required for the structural stability of the enzyme). Single-crossover homologous recombination results in a pseudodiploid configuration with two truncated copies, each of which lacks one of these essential motifs. Oligonucleotides are indicated by horizontal arrows, restriction sites by vertical lines, and DNA probes by horizontal bars. BSD, blasticidin deaminase cassette. (B) PCR analysis of the disrupted locus. Total DNA isolated from cloned, blasticidin-resistant parasites transfected with pCAM-BSD-crkl-3 (transfectant) or from wild-type 3D7 parasites (3D7) was subjected to PCR using the primers indicated (see panel A for their locations). (Left) Primers OL-3 and -4 (diagnostic for the pCAM-BSD-crkl-3 episome or concatemeric inserts); (center) primers OL-1 and -4 (diagnostic for 5' integration); (right) primers OL-3 and -2 (diagnostic for 3' integration). (C) Southern blot analysis. Total DNA was extracted from cloned blasticidin-resistant parasites transfected with pCAM-BSD-crkl-3 (transfectant) and from wild-type 3D7 parasites (3D7) and was digested with PstI and SwaI. (Left) After transfer to a Hybond membrane, the digested DNA was probed with the blasticidin resistance cassette. (Right) The membrane was stripped, and the digested DNA was probed with a *pfcrk-3* amplicon located upstream of the fragment used as an insert in the pCAM-BSD-crkl-3 plasmid. The sizes of comigrating markers are given to the left. The band corresponding to the linearized episome was detected in the transfected parasites (left panel, left lane). The band corresponding to the intact wild-type locus was detected in both the transfected and the untransfected parasites (right panel, both lanes).

signal was detectable in all intraerythrocytic stages of the parasite and largely colocalized with the parasite histone proteins (Fig. 3). In some ring-stage parasites (e.g., the top row in Fig. 3), Pfcrk-3 appears to localize at the periphery of the nucleus, a pattern similar to the “horseshoe” pattern observed by Issar et al. with antibodies against specific histone modifications (24).

Pfcrk-3 is crucial for asexual proliferation. In an attempt to determine whether or not Pfcrk-3 is essential for erythrocytic development, *P. falciparum* 3D7 parasites were transfected

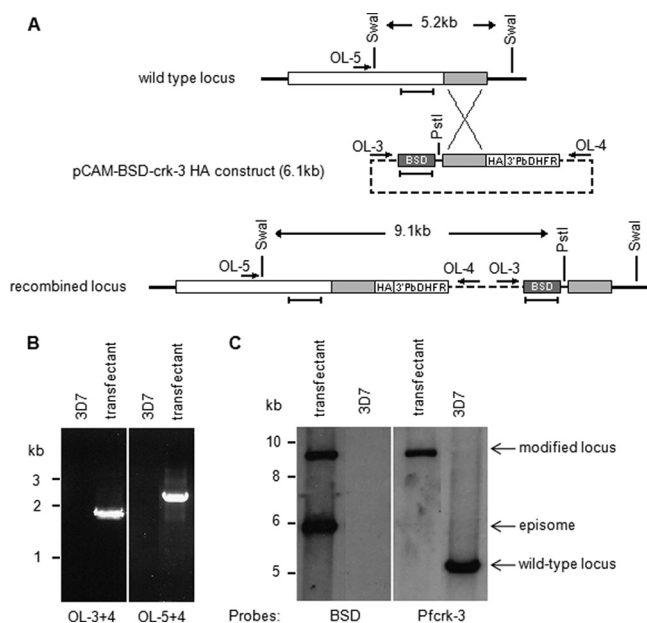


FIG. 5. HA tagging of the *pfcrk-3* locus. (A) Strategy for C-terminal tagging of *pfcrk-3*. The locations of PCR primers used for genotyping are indicated by arrows, restriction sites by vertical lines, and DNA probes by horizontal bars. See the text for details. (B) PCR analysis of HA-tagged locus. Total DNA isolated from blasticidin-resistant parasites transfected with pCAM-BSD-cr3-HA and from wild-type 3D7 parasites was subjected to PCR using the primers indicated. (Left) Primers OL-3 and -4 (diagnostic for pCAM-BSD-cr3-HA episome or concatemeric inserts); (right) primers OL-5 and -4 (diagnostic for 3' integration). (C) Southern blot analysis. Total DNA was extracted from blasticidin-resistant parasites transfected with pCAM-BSD-cr3-HA and from wild-type 3D7 parasites, and 3 μ g was digested with PstI and SmaI, run on a 0.8% agarose gel, transferred to a Hybond membrane, and probed with the blasticidin resistance cassette (see panel A) for the location of the probes, which are indicated by horizontal bars underneath the loci. The band corresponding to the linearized episome or concatemeric insert is detected in the transfected parasites (left panel, lower band). The band corresponding to the wild-type locus (right panel, right lane) is replaced by a larger band of the size expected from the recombination of the plasmid into the locus (both panels, left lanes).

with a pCAM-BSD-based construct containing the central portion of the *pfcrk-3* gene in order to disrupt the locus (Fig. 4A). The resulting blasticidin-resistant parasites were then monitored for integration-specific PCR products. No integration-specific products were observed even after prolonged maintenance of the culture (up to 5 months); the only PCR products obtained corresponded to the unintegrated episome (Fig. 4B). Southern blot analysis confirmed the integrity of the wild-type locus in the transfected cells (Fig. 4C). Failure to disrupt the *pfcrk-3* gene may signify either that the gene is essential for parasite asexual proliferation or that the vector was unable to recombine with the locus. To verify that the *pfcrk-3* locus was indeed accessible to recombination, we attempted to modify the locus without causing loss of function of the gene product. For this purpose, we transfected wild-type parasites with the pCAM-BSD-Pfcrk-3-HA plasmid containing the 3' end of the Pfcrk-3 coding region fused to a hemagglutinin (HA) epitope

followed by the 3' untranslated region (3' UTR) from the *P. berghei dhfr-ts* gene (Fig. 5A). Following single-crossover recombination, we expect an HA-tagged, functional Pfcrk-3 protein to be expressed, but we expect no expression from the wild-type enzyme. PCR analysis of the uncloned blasticidin-resistant population performed 14 weeks after transfection readily detected integration of the construct into the *pfcrk-3* locus (Fig. 5B, primers OL-5 and -4), in addition to the episome (Fig. 5B, primers OL-3 and -4). Cloned lines were obtained by limiting dilution, and PCR examination of the genotypes of individual clones at the *pfcrk-3* locus demonstrated that several clones had lost the wild-type locus (data not shown). The disappearance of the wild-type locus in these clones and the presence of a modified locus of the expected size were verified by Southern blot analysis. Figure 5C shows the data for one such clone, which displays a modified locus and has either retained the episome or integrated concatemers of the plasmid.

Association of Pfcrk-3 with protein kinase and histone deacetylase activities in parasite extracts. In view of (i) the demonstrated role of BUR1 (the enzyme of the yeast kinome that is the closest relative to Pfcrk-3) in the regulation of chromatin modification (including through histone acetylation) (7) and (ii) the emerging importance of histone deacetylases in the regulation of gene expression in *Plasmodium* (see reference 50 for a recent contribution to the field), we set out to investigate whether histone deacetylase activity could be copurified with Pfcrk-3 from *P. falciparum* extracts. Unfortunately, the anti-Pfcrk-3 IgYs did not perform satisfactorily in immunoprecipitation assays (data not shown). We therefore resorted to the parasite line expressing an HA-tagged version of Pfcrk-3 (described in the preceding section). We first verified that an immunoprecipitate obtained with anti-HA antibodies from transgenic, but not from wild-type parasites, contained a protein kinase activity, using mammalian histone H1 (a classical substrate for assaying CDK activity) as a phosphate receiver. Indeed, kinase activity was much higher in the HA immunoprecipitate obtained from parasites expressing HA-tagged Pfcrk-3 than from untransfected 3D7 parasites (Fig. 6A), indicating that Pfcrk-3 is associated, directly or indirectly, with kinase activity (see below). The autoradiogram displays many high-molecular-weight bands in addition to the histone H1 added as a substrate; these may represent copurifying *P. falciparum* proteins (including Pfcrk-3 itself) acting as substrates. We then subjected the HA-immunoprecipitated material to a histone deacetylase activity assay in the presence or absence of NAD as a cofactor. As shown in Fig. 6B, no activity was obtained after immunoprecipitation from extracts of wild-type parasites (bar 1); only samples from parasites expressing the HA-tagged protein (bars 2 to 6) exhibited histone deacetylase activity. Addition of the cofactor resulted in an increase in enzyme activity (compare bars 2 and 3), suggesting the presence of both NAD-dependent and NAD-independent HDAC activities in the Pfcrk-3-associated complexes. This finding is consistent with the activity of recombinant PfSir2 as reported previously (45). Interestingly, the Pfcrk-3-associated HDAC activities were sensitive to the sirtuin inhibitor nicotinamide and the class I/II HDAC inhibitor trichostatin A. Together these data indicate that Pfcrk-3 is part of one or more complexes whose components are capable of protein phosphoryla-

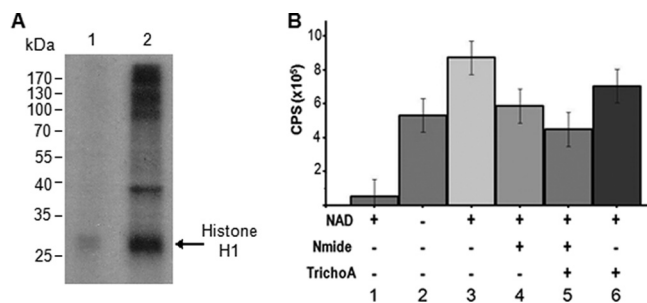


FIG. 6. Immunoprecipitated HA-Pfcrk-3 associates with protein kinase and histone deacetylase activities. (A) Association of HA-Pfcrk-3 with kinase activity. Immunoprecipitates obtained with anti-HA antibodies from untransfected wild-type 3D7 parasites (lane 1) or from parasites expressing HA-tagged Pfcrk-3 (lane 2) were subjected to a kinase activity assay in a standard 30- μ l reaction mixture containing 5 μ g histone H1 as a substrate. The kinase reactions were then run on a 12% polyacrylamide gel, which was dried and subjected to autoradiography. (B) Histone deacetylase assay. Immunoprecipitates obtained with anti-HA antibodies from untransfected wild-type 3D7 parasites (bar 1) or from the HA-tagged Pfcrk3-expressing cell line (bars 2 to 6) were subjected to HDAC assays (Millipore). With the exception of the experiment for which results are shown in bar 2, the immunoprecipitates were incubated for 16 h at 37°C in a substrate buffer containing the sirtuin cofactor NAD. HDAC inhibitors (10 μ M nicotinamide [N-mide] or 2 μ M trichostatin A [TrichoA]) were used separately (bars 4 and 6) or together (bar 5). Reactions were terminated with activator solution followed by fluorescence measurements (excitation wavelength, 360 nm; emission wavelength, 460 nm). Error bars represent standard deviations from duplicate experiments.

tion and histone deacetylation and that it may play a role in chromatin modifications by associating with various HDAC enzymes in *P. falciparum*.

Kinase activity and pulldown assays with recombinant GST-Pfcrk-3. In an attempt to demonstrate that Pfcrk-3 itself possesses kinase activity, a polypeptide containing the catalytic domain plus the C-terminal extension was expressed in *E. coli* as a 100-kDa GST fusion (GST-Pfcrk-3). No kinase activity of the purified recombinant enzyme was observed under our experimental conditions, whereas other recombinant *P. falciparum* kinases were active (data not shown). We reasoned that activity of GST-Pfcrk-3 might require interaction with a cyclin-like activator. Four plasmodial cyclin-like proteins have been cloned in our laboratory (Pfcyc-1, Pfcyc-2, Pfcyc-3, and Pfcyc-4), two of which (Pfcyc-1 and Pfcyc-3) activate recombinant PfPK5 (another CDK-related enzyme) *in vitro* (38). Incubation of Pfcrk-3 with these four different recombinant cyclins did not cause activation of the recombinant enzyme, nor did addition of RINGO, a potent activator of some CDKs, including PfPK5 (32, 42) (data not shown).

The absence of activity of the recombinant Pfcrk-3 catalytic domain even in the presence of cyclins may result from the fact that the fusion protein lacks the large N-terminal extension, or from the absence of additional activator mechanisms, such as phosphorylation by other kinases. To address the latter issue, we repeated the kinase assay following incubation of recombinant Pfcrk-3 in parasite extracts. Pulldown experiments, in which glutathione-agarose beads loaded with GST-Pfcrk-3 were incubated in extracts from asynchronous parasites, washed, and subjected to *in vitro* kinase activity assays, allowed us to detect histone H1 kinase activity associated with the

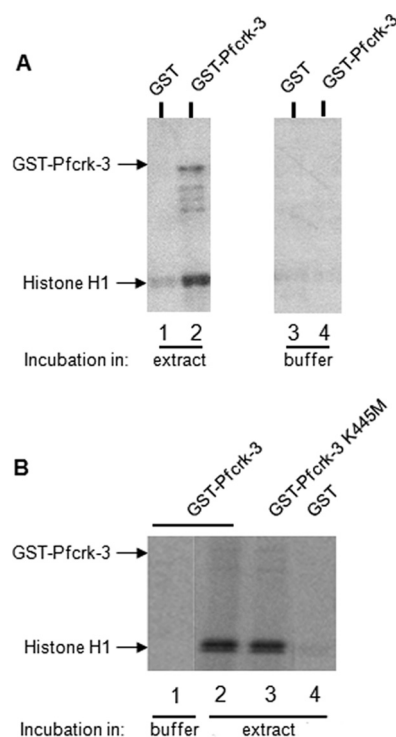


FIG. 7. Association of recombinant GST-Pfcrk-3 with histone H1 kinase activity. (A) Pulldown experiments using wild-type GST-Pfcrk-3. One microgram of GST-Pfcrk-3 (lanes 2 and 4) or GST alone (lanes 1 and 3) bound to glutathione-agarose beads was incubated in parasite extract (lanes 1 and 2) or in parasite lysis buffer as a negative control (lanes 3 and 4). The beads were washed and resuspended in kinase assay buffer, and *in vitro* kinase assays were performed by addition of a reaction mixture containing radiolabeled ATP and histone H1. (B) Pulldown experiments using wild-type and kinase-dead GST-Pfcrk-3, followed by kinase assays. Portions (0.5 μ g) of GST-Pfcrk-3 (lanes 1 and 2), GST-Pfcrk-3-K445M (lane 3), or GST alone (lane 4) bound to glutathione-agarose beads were incubated in parasite extracts (lanes 2 to 4) or in parasite lysis buffer as a negative control (lane 1). The beads were washed, and *in vitro* kinase assays were performed by addition of a reaction mixture containing the radiolabeled ATP and histone H1.

recombinant enzyme (Fig. 7A, lane 2). Furthermore, under these conditions, we also observed a weak signal at approximately 100 kDa, which is likely to be GST-Pfcrk-3 itself. A much weaker signal was detected when the pulldown from the parasite extract was performed with the GST moiety alone (Fig. 7A, lane 1), and no signal was observed when GST-Pfcrk-3 was incubated in parasite lysis buffer that did not contain parasite proteins (lane 4). The activity observed in Fig. 7A, lane 2 (phosphorylation of histone H1 and of GST-Pfcrk-3), might result either from activation of Pfcrk-3 itself by a component of the pulled complex (e.g., through binding of a cyclin-like regulator or through a phosphorylation event) or from another protein kinase that had been pulled down by GST-Pfcrk-3. To distinguish between these possibilities, we repeated the pulldown experiment using a kinase-dead mutant of GST-Pfcrk-3 (GST-Pfcrk-3-K445M) in which a conserved lysine residue involved in ATP orientation was replaced by a methionine residue (Fig. 7B). This yielded a signal of an intensity similar to that obtained when the pulldown was performed with the wild-type enzyme (Fig. 7B, lanes 2 and 3), suggesting that another

plasmodial protein kinase that interacts with Pfcrk-3 was responsible for at least a fraction of the detected kinase activity identified in Fig. 7A.

Pulldown experiments thus demonstrate that Pfcrk-3 is associated with kinase activity in parasite extracts and that at least part of this activity is due to another kinase that interacts with Pfcrk-3; we favor the hypothesis that Pfcrk-3 itself also possesses activity *in vivo* (see Discussion).

DISCUSSION

Bioinformatics considerations. BLASTP analysis showed that the Pfcrk-3 kinase domain displays maximal homology to BUR1 (BLASTP score, 164; E-value, $1e-40$), a yeast transcriptional CDK-related kinase previously described as SGV1 and required for recovery from mating pheromone-induced cell cycle arrest (23). When complexed to its cyclin partner, BUR2, BUR1/SGV1 phosphorylates the carboxy-terminal end of RNA polymerase II (40, 54, 55) and other transcription-related substrates (28) and regulates trimethylation (30). Thus, the BLASTP results are fully consistent with the phylogenetic analysis in which Pfcrk-3 clustered with the transcriptional CDKs, which include SGV1 and human CDK9 (Fig. 1). Another feature shared by Pfcrk-3 and BUR1 is a long C-terminal extension that is usually not found in other CDKs (including mammalian CDK9); remarkably, this extension has exactly the same size (335 amino acids) in both enzymes. Even though the Pfcrk-3 protein appears to be processed during parasite development (Fig. 2B), the C-terminal extension is presumably maintained, since the C-terminally HA-tagged enzyme can be recovered via the tag (Fig. 6).

Many CDKs are negatively regulated by phosphorylation of Thr14 and Tyr15 (human CDK2 numbering), of which only Tyr15 is conserved in Pfcrk-3. Interestingly, the inverse configuration is observed in BUR1 and CDK9, where only Thr14 is conserved; this may indicate different modes of regulation between Pfcrk-3 and transcriptional CDKs in Opisthokonts (the phylum including yeast and metazoans). In contrast, the conservation in Pfcrk-3, yeast BUR1, and human CDK9 of Thr160, the target of activating phosphorylation by CDK-activating kinases (CAKs), is consistent with the observation that BUR1 is activated by CAKs (55) and suggests that a similar mechanism may regulate Pfcrk-3 activity.

Pfcrk-3 function. Successful *in situ* 3' HA tagging (Fig. 5) establishes that the *pfcrk-3* locus is recombinogenic, strongly suggesting that the lack of success in obtaining parasites with a disrupted locus is due to the fact that the gene is crucial for asexual proliferation. The possibility remains that Pfcrk-3 inactivation is not strictly lethal but causes a growth rate defect that renders parasites unable to compete with parasites that retain a wild-type locus in the transfected population. In this context, it is noteworthy that in addition to the inability of BUR1 mutants to recover from mating pheromone-dependent cell cycle arrest (23), normal growth under various conditions is affected in BUR1 and BUR2 mutants (54).

Thus, bioinformatics analyses, subcellular localization, and association with histone deacetylase activity all concur to assign Pfcrk-3 as a chromatin-associated CDK involved in the regulation of transcription. It would be of great interest, in order to gain further insight into the precise function of Pfcrk-3, to identify

which of the several putative HDACs (one class I HDAC, two class II HDACs, and two class III sirtuins [44]) encoded by the *P. falciparum* genome is responsible for the activity we observed to be associated with HA-Pfcrk-3. Sensitivity to nicotinamide suggests that a class III HDAC, such as Pfsir2, is involved (45), but caution must be exercised until the enzyme is experimentally identified. The parasites expressing HA-tagged Pfcrk-3 represent a tool that can now be used for affinity chromatography/mass spectrometry-based identification of this and other components of the protein complex that includes Pfcrk-3. Further information on the role of Pfcrk-3 in gene expression could be gained by performing high-resolution colocalization studies to determine whether the spatial distribution of Pfcrk-3 correlates with that of specific histone modifications, as hinted by the "horseshoe" appearance of the protein distribution pattern in ring-stage parasites (Fig. 3) (24).

A recombinant protein containing the catalytic domain and the C-terminal extension, but lacking the N-terminal extension, displayed no enzymatic activity. This is expected for a CDK homologue; for example, no activity was observed with the PpPK5 CDK in the absence of a cyclin (or equivalent) activator (32). However, in contrast to what we observed with PpPK5, addition of recombinant cyclins to the reaction mixture did not result in Pfcrk-1 activation. We showed that a histone H1 kinase activity can be pulled down from parasite extracts using recombinant Pfcrk-3; kinase activity was also recovered when a kinase-dead mutant was used in the pulldown assay, suggesting that another protein kinase is present in a complex that includes Pfcrk-3. Taken together, our data are consistent with the proposition that Pfcrk-3 functions in a large complex regulating transcription. In other systems, it is well established that transcriptional complexes contain several PKs, including CDKs. If, as we suspect in view of the conservation of all residues that are important for activity, Pfcrk-3 is indeed an active CDK, it will of course be crucial for our understanding of its function to identify substrates of the enzyme. The present study generated the tools necessary to proceed with investigations in these areas and constitutes a solid basis for further work aimed at understanding the control of gene expression in malaria parasites.

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